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Rapid and Quantitative Detection of Aspartic Proteinase in Animal Tissues by Radio-labeled Pepstatin A

Masanori Mukai[1], Toshihiko Kondo[2] and Katsutoshi Yoshizato[1,3]

[1]*Molecular Cell Science Laboratory, Department of Biological Science, Faculty of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashihiroshima-shi, Hiroshima 724 and* [2]*Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Maebashi 371, Japan*

ABSTRACT—A new radio-derivative of pepstatin A was developed and was shown to be used as a probe for rapidly and quantitatively detecting aspartic proteinases in animal tissues. The carboxyl group of pepstatin A was activated by the water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and was then coupled with N-hydroxysulfosuccinimide (Sulfo NHS). [^{35}S]-Methionyl-pepstatin with a relatively high specific activity was obtained by coupling the Sulfo NHS-pepstatin with L-[^{35}S]-methionine. Binding specificity of the pepstatin A derivative was characterized using pepsin as a test enzyme. Binding experiments showed that the radio-labeled pepstatin can be used as a probe which binds specifically to aspartic proteinases and detects them. The probe could detect as low as 0.1 nmoles of pepsin. To know whether the radio-labeled probe can be actually used to detect aspartic proteinases in animal tissues, it was applied to the tail tissue of metamorphosing amphibian tadpole which had been known to show high activity of cathepsin D. The assay demonstrated marked increase in aspartic proteinases at the climax stage. It was concluded that the radio-labeled pepstatin derivative developed by the present study is useful for quick and quantitative determination of pepstatin-reactive enzymes in animal tissues.

INTRODUCTION

The aspartic proteinase is a group of proteinases (EC 3.4. group 23) which has an optimum pH at an acidic region. The family contains pepsin, cathepsin D, cathepsin E, renin, chymosin and gastricsin. Amino acid sequences of mammalian aspartic proteinases and the corresponding nucleotide sequences of their cDNA clones revealed that they have the active center, which contains two aspartic residues and are highly conserved among aspartic proteinases [4, 8, 10, 12, 32, 33, 35].

Pepstatin A is produced by actinomyces [37] and is a potent inhibitor of pepsin ($Ki=4.5\times10^{-11}$ M), cathepsin D ($Ki=10^{-10}$ M) and other aspartic proteinases [3, 27]. Pepstatin A binds to the active center of the enzyme in a stoichiometric manner which is surrounded by two aspartic moieties [3, 19, 37].

Radio-labeled pepstatin derivatives have been chemically synthesized. Pepstatin was coupled with [^{3}H]-glycine and used for determining Kd values of a complex of pepstatinyl-[^{3}H]-glycine-cathepsin D [15]. A radio-iodinated derivative of pepstatin was prepared by introducing a tyrosine residue which was then iodinated with ^{125}I. This was utilized for the determination of Kd of pepsin-pepstatin-[^{125}I]-monoiodotyrosine methyl ester complex [41]. However, ^{3}H-labeled compound is chemically unstable because tritium is exchangeable with surrounding hydrogen. ^{125}I-Labeling has disadvantages because of its short half life of radioactivity.

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Chemical modification of pepstatin has been designed to utilize the inhibitor as an experimental probe which tightly binds to the active site of aspartic proteinases and carries reporter groups [16]. Bimane-labeled pepstatin [21] and dinitrophenyl-pepstatin [22] have been synthesized and used for the determination of the subcellular location of cathepsin D in cultured human synovial cells. These methods of modifications require relatively complicated techniques of organic chemistry and contain multiple steps including techniques of column chromatography. Because both pepstatin derivatives described above are detected by fluorescence, these derivatives cannot be used as a probe in cases where samples to be analyzed contain fluorescent materials.

In the present study the authors aimed at developing a much simple radio-labeling procedure of pepstatin to obtain a stable derivative. For this purposes, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) [42] and N-hydroxy-sulfosuccinimide (Sulfo NHS) [2, 34] were selected as activating agents since they are easy to handle because of their hydrophilicity. The activated pepstatin was coupled with L-[^{35}S]-methionine which has longer half life of decay than ^{125}I.

In the amphibian metamorphosis, the larval tail tissue is subject to the histolysis by several proteinases such as collagenase [11, 25] and cathepsin D [18, 29, 38]. This phenomenon was utilized to see validity and usefulness of the radio-labeled pepstatin derivative for detecting proteinases in animal tissues. The present study succeeded in demonstrating that the radio-labeled pepstatin is a useful probe for quick and quantitative detection of aspartic proteinases in animal tissues.

MATERIALS AND METHODS

Materials

Pepstatin A was purchased from Peptide Institute, Inc. (Osaka, Japan). EDC and Sulfo NHS were obtained from Pierce (Rockford, IL). L-[^{35}S]-methionine was from New England Nuclear (Boston, MA). SEP-PAK C_{18} was from Waters (Milford, MASS). Pepsin was a product of Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical grade. Tadpoles of bullfrog, *Rana catesbeiana*, were purchased from a local animal supplier. They were staged as described by Taylor and Kollros (TK stage) [36].

Coupling reaction

Pepstatin A (0.7 mg, 1 μmoles) was dissolved in 200 μl of dimethyl sulfoxide. Sulfo NHS (10 μmoles) and EDC (100 μmoles) were dissolved in 800 μl of 16 mM sodium phosphate buffer, pH 7.4, containing 20% dimethyl sulfoxide. These were mixed together and were incubated at room temperature (ca. 20°C) for 1 hr. The product of this reaction, Sulfo NHS-pepstatin, was diluted with 4 ml of 20 mM sodium phosphate buffer, pH 7.4, and was applied slowly to a SEP-PAK C_{18} column (1 drop/sec) using a syringe. The column was washed twice with 5 ml of 20 mM sodium phosphate buffer, pH 7.4, for removing excess amounts of activating agents (Sulfo NHS and EDC). Sulfo NHS-pepstatin was eluted from the column twice with 2 ml of dimethyl sulfoxide, mixed with 4 ml of 10 mM sodium phosphate buffer, pH 7.4, containing L-[^{35}S]-methionine (100 pmoles, 4.1 MBq, 1.6×10^8 cpm) and was incubated at room temperature (ca. 20°C) for 2 hr. The reaction mixture in which [^{35}S]-methionyl-pepstatin was produced was diluted with deionized water and loaded on a SEP-PAK C_{18} column in the same way as described above. The column was washed twice with 10 ml of deionized water. This step separated the labeled pepstatin from uncoupled L-[^{35}S]-methionine and other by-products which did not covalently bind to pepstatin A. The eluate containing [^{35}S]-methionyl-pepstatin (0.72 MBq, 2.8×10^7 cpm/4 ml dimethyl sulfoxide) was kept at −20°C before use.

Separation of [^{35}S]-methionyl pepstatin A from free pepstatin A

[^{35}S]-Methionyl pepstatin prepared as described above still contained excess amounts of pepstatin A. Free pepstatin A was removed mainly by HPLC as follows. The preparation was 4-fold diluted with deionized water and loaded on a SEP-PAK C_{18} column which was then eluted with acetonitrile. The eluate containing pepstatin A and its [^{35}S]-methionyl derivative was evaporated to dryness at 40°C *in vacuo* and was dissolved in an appropriate amount of dimethyl sulfoxide. The concentrated radio-labeled pepstatin A was subjected to reversed phase high-performance liquid chromatography (HPLC, Inertsil 300-C_8, 4.6×100 mm, Gasukuro Kogyo Inc. Tokyo, Japan) and eluted at 50°C with a linear gradient of acetonitrile (from 25 to 55%) containing 0.1% trifluoroacetic acid (TFA). The flow rate was 0.4 ml/min. The eluate was monitored by 220 nm for pepstatin A and by the radioactivity for the labeled one. Radioactive fractions were collected and concentrated by evaporation.

Binding assays

Appropriate amounts of pepsin (10–440 μg/20 μl H_2O/membrane) were spotted on nitrocellulose membranes (1.5×1.5 cm^2). Membranes were dried and incubated in 20 mM sodium acetate buffer, pH 5.0, containing 1% bovine serum albumin (BSA) for 10 min on ice for protecting nonspecific binding of pepstatin A to the membranes. Then, the blocking buffer was replaced with 2 ml of 20 mM sodium acetate buffer, pH 5.0, which contained radio-labeled pepstatin A ([^{35}S]-methionyl-pepstatin A, 10^4–10^6 cpm/assay). The membranes were incubated for additional 30 min on ice. Membranes were then washed 4 times on ice with 2 ml of 20 mM sodium acetate buffer, pH 5.0, to remove radio-labeled pepstatin A that had not bound to pepsin, and were dried and placed in 5 ml of a scintillation cocktail (Atomlight, New England Nuclear, Boston, MA) to count radioactivities using a liquid scintillation counter (LSC-3000, Aloka). Nonspecific binding of radio-labeled pepstatin A to the nitrocellulose membrane was estimated through the identical procedure described above with an exception that membranes were washed with neutral buffer (20 mM sodium phosphate buffer, pH 7.4).

Binding of [^{35}S]-methionyl-pepstatin to the tail tissue of bullfrog tadpole

The tail of tadpole of bullfrog, *Rana catesbeiana*, was used as a source of aspartic proteinases for the binding assay of [^{35}S]-methionyl-pepstatin A. Tails were dissected from bullfrog tadpoles at the premetamorphic stage (TK stage XVII) or the climax stage of metamorphosis (TK stage XXIII) and kept at −20°C until use. The frozen tail tissues were defrosted on ice and finely minced to small pieces (about 3×3×3 mm^3) at 0–4°C with scissors in the solution containing 0.1 M NaCl and 20 mM sodium acetate buffer, pH 5.0. Tissue pieces were collected in microfuge tubes (Ca. 100 mg wet weight tissue/tube) and suspended in 1 ml of 20 mM sodium acetate buffer containing 0.1 M NaCl at 0–4°C. The radio-labeled pepstatin A (2.0×10^4 cpm/assay) was added to the tissue suspension and allowed to stand for binding at 0–4°C for 30 min. For removal of unbound pepstatin probe, tubes were then centrifuged at 1,700×*g* for 5 min at 4°C and the supernatants were discarded. The precipitates were washed 3 times with 1 ml of 20 mM sodium acetate buffer containing 0.1 M NaCl, pH 5.0. Then, for recovering radio-labeled pepstatin A bound to tissues, precipitates were lysed in 600 μl of 0.1 N NaOH at 37°C for 2 hr and were centrifuged at 7,000×*g* for 10 min at room temperature. Radioactivity in the supernatant was counted. The protein concentration of lysates was determined by the procedure described by Lowry *et al.* using BSA as a standard [20].

RESULTS

The carboxyl group of pepstatin A was activated by EDC to form o-acylurea which was then coupled with Sulfo NHS. Sulfo NHS-pepstatin thus obtained was more resistant to hydrolysis than o-acylurea and was hydrophobic. Excess amounts of hydrophilic activating reagents were easily removed from the reaction mixture using a reversed phase column of SEP-PAK C_{18}. Coupling of the activated pepstatin A with the amino group of L-[^{35}S]-methionine produced [^{35}S]-methionyl-pepstatin A with specific activity of 0.72 MBq/μmole (2.8×10^7 cpm/μmole).

Specificity of the radio-labeled pepstatin A was characterized by analyzing its binding properties to pepsin that had been immobilized on a nitrocellulose membrane. The radio-labeled pepstatin was bound to the enzyme at pH 5.0, but not at pH 7.4, same as pepstatin A (Fig. 1). The binding of

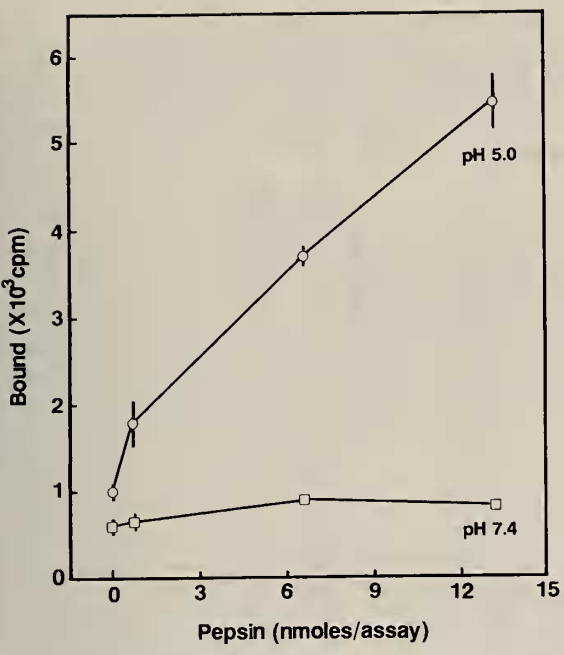

FIG. 1. pH-Dependent binding of [^{35}S]-methionyl-pepstatin A to pepsin. The radio-labeled derivatives (3.6×10^4 cpm/assay) were incubated with various amounts of pepsin (0.7–14.6 nmoles) that had been dotted on nitrocellulose membranes. For removing unbound pepstatin A probes, membranes were washed with 20 mM sodium acetate buffer, pH 5.0, (circle) or with 20 mM sodium phosphate buffer, pH 7.4, (square). Each point represents the mean of triplicate assays and bars indicate standard errors of the mean.

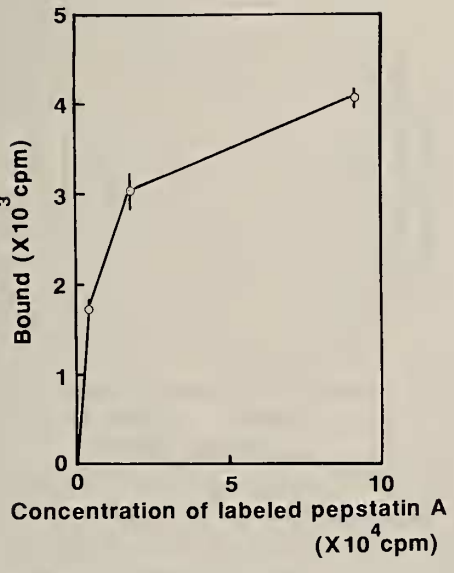

FIG. 2. Binding of [^{35}S]-methionyl-pepstatin A to the fixed amount of pepsin. Pepsin (5.7 nmoles) was immobilized on a nitrocellulose membrane (1.5×1.5 cm^2) and incubated with indicated amounts of labeled pepstatin A (4.4×10^3–8.8×10^4 cpm/assay). Radioactivity bound to membranes was counted as amounts of labeled pepstatin A bound to pepsin. The radioactivity of 10^3 cpm corresponds to 35 pmoles of the pepstatin A probe. Each point represents the mean of triplicate assays. Bars indicate standard errors of the mean.

[^{35}S]-methionyl-pepstatin A to pepsin is proportional to pepsin concentrations in the range of 1–12 nmoles. Radio-labeled pepstatin A bound increasingly to the fixed amount of pepsin as the amount of labeled pepstatin increased up to around the dose of 2×10^4 cpm and then the binding was leveled off (Fig. 2), suggesting that the labeled pepstatin A binds to limited and specific sites of pepsin.

Binding of [^{35}S]-methionyl-pepstatin A to pepsin was competitively suppressed by pepstatin A (Fig. 3), indicating that the radio-labeled derivative shows the same binding specificity as pepstatin A.

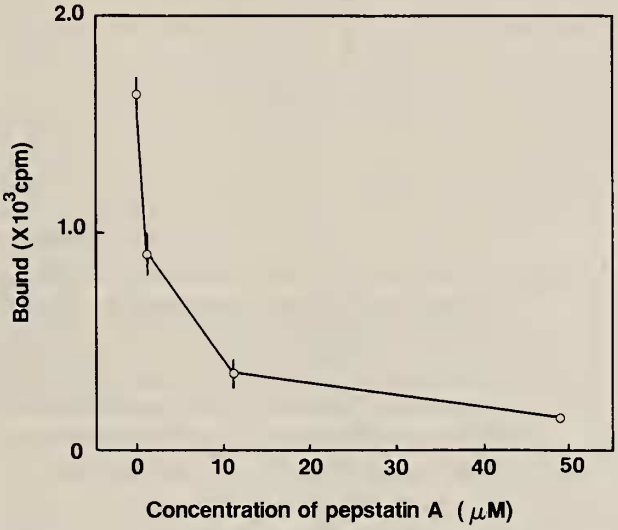

FIG. 3. Suppression of the binding of the radio-labeled pepstatin A to pepsin by pepstatin A. Pepsin (5.7 nmoles/assay) spotted on membranes was incubated with the fixed amount of the pepstatin derivatives (8.8×10^3 cpm/assay) and varied amounts of pepstatin A (0–48 μM) as a competitor. The radioactivity bound to pepsin was counted. Each point represents the mean of triplicate assays with its standard error indicated by a bar.

Figure 1 shows that the [^{35}S]-methionyl-pepstatin A prepared as above can detect pepsin when its amount is more than 1.0 nmoles. To get the probe for aspartic proteinases showing a higher specific radioactivity, the labeled pepstatin A was further purified by subjecting it to reversed phase HPLC (Fig. 4). Pepstatin A was eluted at 38% acetonitrile as a single peak. The radio-labeled pepstatin A was eluted at 44% acetonitrile also as a single peak. The pepstatin derivative thus purified had a specific activity of 3.6 MBq/μmole and could detect as low as 0.1 nmoles of pepsin. Its binding to pepsin was proportional to the concentration of the enzyme in the range of 0.1–0.3 nmoles (Fig. 5).

The radio-labeled pepstatin A was tried to use as a probe to detect aspartic proteinases in the animal tissue. The metamorphosing tadpole tail was quantitated for the pepstatin-reactive enzymes (Fig. 6). The specific binding of the probe to the tail of a metamorphosing tadpole was 18-fold higher than the binding to the tail of a premetamorphic animal. If we postulate that all the bound probe recovered

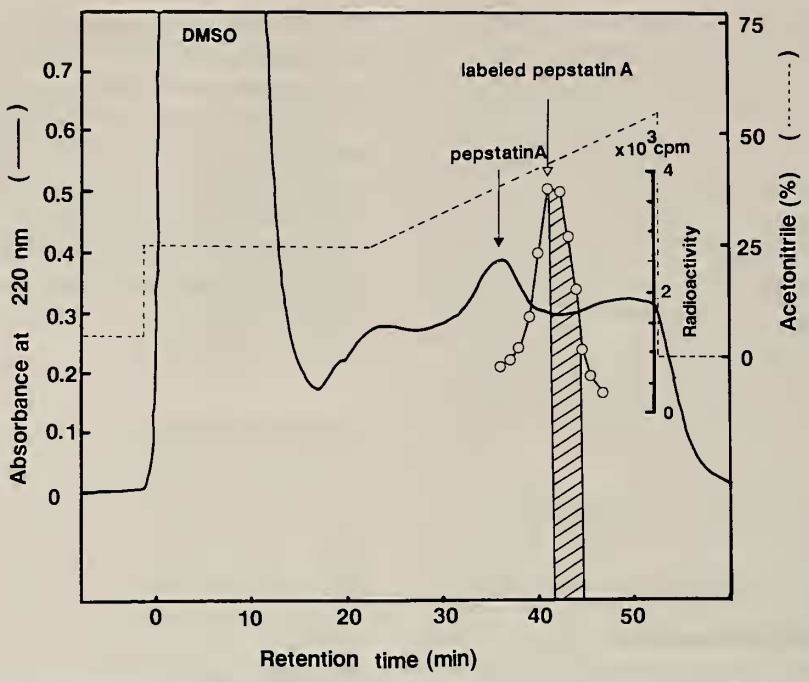

FIG. 4. Separation of [^{35}S]-methionyl-pepstatin A and pepstatin A by reversed phase HPLC. The radio-labeled pepstatin A fraction obtained at the second reaction was applied to a Inertsil 300-C_8 (4.6×100 mm) and eluted at 50°C with a linear gradient of 25–55% of acetonitrile (dotted line) containing 0.1% TFA. The flow rate was 0.4 ml/min. The solid line indicates the absorbance at 220 nm. The radioactivity of each fraction (0.4 ml) was counted (circle). The first peak of 220 nm contains dimethyl sulfoxide which was used as the solvent of pepstatin A. The closed arrow at 38% acetonitrile shows the peak of pepstatin A and the open arrow at 44% acetonitrile the peak of [^{35}S]-methionyl-pepstatin. The shaded fractions were collected as [^{35}S]-methionyl pepstatin and used as a probe for aspartic proteinases (3.6 MBq/μmole).

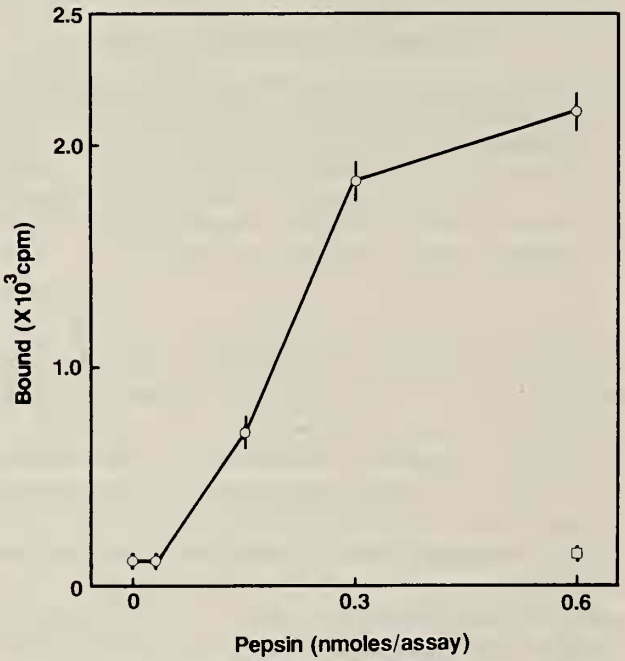

FIG. 5. Binding of the [^{35}S]-methionyl-pepstatin A to pepsin. HPLC-purified radio-labeled pepstatin A (10^4 cpm/assay) was incubated for 30 min at 0–4°C with nitrocellulose membranes containing varied amounts of pepsin. Membranes were washed with acidic buffer and counted for radioactivity (circle). Non-specific binding to nitrocellulose membranes was obtained as the radioactivity remaining on membranes when they were washed with neutral pH (square). The radioactivity of 10^3 cpm is equivalent to 7.1 pmoles of the pepstatin A probe. Each point represents the mean of triplicate assays and bars indicate standard errors of the mean.

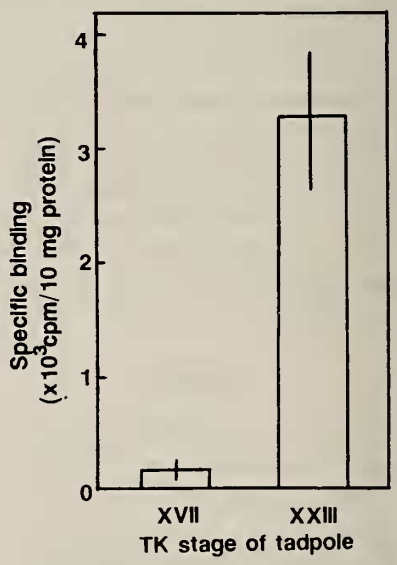

FIG. 6. Detection of aspartic proteinase in the tail of tadpole by the [^{35}S]-methionyl-pepstatin A. The specific binding of the probe was calculated by subtracting the value of radioactivity obtained in the presence of excess amounts of pepstatin A (30 μM) from that without it. Tail tissues of bullfrog tadpole (about 100 mg wet weight/assay) at premetamorphosis (TK stage XVII) or the climax of metamorphosis (TK stage XXIII) were incubated with the radio-labeled pepstatin A (HPLC-purified, 2.0×10^4 cpm/assay) in solution of 20 mM sodium acetate buffer and 0.1 M NaCl, pH 5.0, with or without a competitor, pepstatin A (30 μM). The radioactivity of 10^3 cpm is equivalent to 7.1 pmoles of the pepstatin probe as in Fig. 5. Each value represents the mean of triplicate assays with its standard error indicated by a bar.

from tissues, the tail contained binding sites of 0.7 nmoles/10 mg protein at the climax stage and 0.04 nmoles/10 mg protein at the premetamorphic stage. These values of binding sites are expressed as those for "pepsin equivalent".

DISCUSSION

Pepstatin A is a proteinase inhibitor that is not specific to one species of enzyme but shows the activity toward several species of enzymes grouped as the aspartic proteinase family including pepsin, cathepsin D, cathepsin E, renin, chymosin and gastricsin. Therefore, [^{35}S-]-methionyl-pepstatin A developed in the present study can be applied to animal tissue for the first screening of proteinases which is grouped into this family.

The radio-labeled pepstatin A keeps the same activity as pepstatin A. We used the carboxyl group of pepstatin A as the site of modification, as pepstatin-conjugated resins have been usually prepared by modifying this residue and utilized for purification of aspartic proteinases such as cathepsin D [1]. The present study also confirms that the carboxyl group of pepstatin can be modified as a connecting site for reporter groups without destroying properties which are required for the inhibitor to bind aspartic proteinases. Radioactive methionine can be successfully introduced by this modification into pepstatin A.

Since this additional chain of methionine is neutral in charge, it does not disturb the ionic condition of the binding site of aspartic proteinases. The [^{35}S]-methionyl-pepstatin A we prepared is more hydrophobic than pepstatin A, which may help the derivative to approach more easily for the binding site of the enzymes than pepstatin A because the site is thought as a hydrophobic pocket in pepsin [27].

Pepstatin A binds to aspartic proteinases in a stoichiometric manner. The present study shows that the binding of the radio-labeled pepstatin A to pepsin is proportional to concentrations of pepsin, indicating that it can be used as a sensitive probe for the quantitative analysis of aspartic proteinases. Utilizing this probe, we could detect 0.1 nmoles of pepsin. It seems that other methods such as radio-immuno assay are more sensitive than the method described in the present study. However, the radio-labeled pepstatin A is much useful as compared to the antibody against aspartic proteinases. We have to prepare the specific antibody in each case of the study against the enzyme of target. In contrast, the radio-labeled pepstatin A can be used for detecting the enzyme belonging to the family of aspartic proteinases.

We made some alterations in the assay of binding of the pepstatin A derivative when we tried to detect aspartic proteinases in tadpole tissues. Binding was assayed on nitrocellulose membranes for the test enzyme (pepsin), while the assay for the tissue sample was done in tissue pieces suspended in acid solution. The pepstatin A derivative bound to enzymes in the tissue was then removed by centrifugation in alkaline condition. The reason for this alteration was the relatively low capacity of nitrocellulose membranes to hold tissue proteins. We first tried to assay enzymes in the tissue samples using nitrocellulose membranes as we did for the test enzyme. However, reliable and reproducible values of binding of the radio-labeled pepstatin A could not be obtained in the membrane assay.

This probe is shown to detect pepstatin-sensitive proteinase(s) in the tail of bullfrog tadpole. The amount of specific binding to the tail tissue much increases at the climax stage of metamorphosis (TK stage XXIII) as compared to the premetamorphic one. Several reports demonstrated that cathepsin D like proteinase activity in the tail increases at the climax of metamorphosis [9, 18, 29, 38, 39, 43] and pepstatin sensitive proteinase plays important roles in the regression of tail during metamorphosis [31]. Pepstatin A binding sites at the climax stage of metamorphosis increases 18-fold as compared to those at the premetamorphic stage. The ratio of cathepsin D activity of the tail at the climax stage to that at the premetamorphic stage is 18 in *Rana catesbeiana* [29] and 16 in *Xenopus laevis* [39]. These values are very close to that obtained by our method.

Pepstatin-sensitive proteinases are found also in a wide variety of life including vertebrates such as human [5], bovine [30], porcine [14], rabbit [6], rat [10], chicken [5, 26], frog [23] and fish [7], and invertebrates such as marine mussel [24] and hemipteran insect [13]. Plant [28], bacteria [17] and retrovirus [40] have also been reported to contain pepstatin-sensitive proteinases. Therefore, it is considered that aspartic proteinases might play fundamental roles in metabolic processes of varieties of life. However, the information on the enzyme other than mammalian origin has been poor. There is a possibility that the binding site recognized by pepstatin A is highly conserved in the molecular evolution of aspartic proteinases. The pepstatin derivative reported here is expected to be useful in detecting unknown aspartic proteinases in the wide range of life and studying them from a comparative point of view.

It has been shown that aspartic proteinase of HIV virus can be converted by the gene technology into a mutant enzyme that has no enzymatic activity but can bind to pepstatin A [40]. This indicates that the catalytic site of the enzyme is different from the pepstatin binding site, although both sites are in the active center. The radio-active pepstatin developed in the present study is expected to be useful in detecting enzymes of the aspartic proteinase family that lose their catalytic activity.

We could not develop pepstatin A derivative which shows higher sensivity of detection than the method to directly measure the enzyme activity or conventional immunological detection method using specific antibody. It remains as a future study to develop a method to prepare [^{35}S]-methionyl-pepstatin A with much more higher specific activity.

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REFERENCES

1 Afting EG, Becker ML (1981) Two-step affinity-chromatographic purification of cathepsin D from pig myometrium with high yield. Biochem J 197: 519–522

2 Anjaneyulu PSR, Staros JV (1987) Reactions of N-hydroxysulfosuccinimido active esters. Int J Pep Pro Res 30: 117–124

3 Aoyagi T, Morishima H, Nishikawa R, Kunimoto S, Takeuchi T, Umezawa H, (1972) Biological activity of pepstatins, pepstanone A and partial peptide on pepsin, cathepsin D and renin. J Antibiot 25: 689–694

4 Azuma T, Liu W, Laan DJV, Bowcock AM, Taggart RT (1992) Human gastric cathepsin E gene. J Biol Chem 267: 1609–1614

5 Barrett AJ (1970) Cathepsin D, purification of isoenzymes from human and chicken liver. Biochem J 117: 601–607

6 Barrett AJ (1971) Purification and properties of cathepsin D from liver of chicken, rabbit and man. In "Tissue proteinases" Ed by Barrett AJ, Dingle JT North-Holland Publishing Co., Amsterdam, pp 109–133

7 Bonete MJ, Manjon A, Llorca F, Iborra JL (1984) Acid proteinase activity in fish II. Purification and characterization of cathepsins B and D from *Mujil auratus* muscle. Comp Biochem Physiol 78B: 207–213

8 Faust PL, Kornfeld S, Chirgwin JM (1985) Cloning and sequence analysis of cDNA for human cathepsin D. Proc Natl Acad Sci USA 82: 4910–4914

9 Fujii Y, Taguchi M, Kobayashi K, Horiuchi S (1991) Immunochemical studies on cathepsin D like enzyme in the tadpole tail of *Rana catesbeiana*. Zool Sci 8: 511–520

10 Fujita H, Tanaka Y, Noguchi Y, Kona A, Himeno M, Kato K (1991) Isolation and sequencing of a cDNA clone encoding rat liver lysosomal cathepsin D and the structure of three forms of mature enzymes. Biochem Biophys Res Commun 179: 190–196

11 Gross J, Lapiere CM (1962) Collagenolytic activity in amphibian tissues : A tissue culture assay. Proc Natl Acad Sci USA 48: 1014–1022

12 Hobart PM, Fogliano M, O'Connor BA, Schaefer IM, Chirgwin JM (1984) Human renin gene : Structure and sequence analysis. Proc Natl Acad Sci USA 81: 5026–5030

13 Houseman JG, Downe AER (1983) Cathepsin D-like activity in the posterior midgut of hemipteran insect. Comp Biochem Physiol 75B: 509–512

14 Huang JS, Huang SS, Tang J (1979) Cathepsin D isozymes from porcine spleens. J Biol Chem 254: 11405–11417

15 Knight CG, Barrett AJ (1976) Interaction of human cathepsin D with the inhibitor pepstatin. Biochem J 155: 117–125

16 Knight CG, Hornebeck W, Matthews ITW, Hembry RM, Dingle JT (1980) Interaction of dinitrophenyl-pepstatins with human cathepsin D and with anti-dinitrophenyl antibody. Biochem J 191: 835–843

17 Kobayashi H, Kusakabe I, Murakami K (1982) Rapid isolation of microbiral milk-clotting enzymes by N-acetyl(or N-isobutyryl)-pepstatin-aminohexyl agarose. Anal Biochem 122: 308–312

18 Kobayashi K, Hara M, Horiuchi S (1985) Isolation of lysosomes from the tail of metamorphosing bullfrog tadpole. Comp Biochem Physiol 81B: 603–607

19 Kunimoto S, Aoyagi T, Nisizawa R, Komai T, Takeuchi T, Umezawa H (1974) Mechanism of inhibition of pepsin by pepstatin II. J Antibiot 27: 413–418

20 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275

21 Matthews ITW, Decker RS, Knight C G (1981) Bimane-labelled pepstatin, a fluorescent probe for the subcellular location of cathepsin D. Biochem J 199: 611–617

22 Matthews ITW, Decker RS, Hornebeck W, Knight CG (1983) Dinitrophenyl-pepstatins as active site directed localization reagents for cathepsin D. Biochem J 211: 139–147

23 Nanbu M, Kobayashi K, Horiuchi S (1988) Purification and characterization of cathepsin D-like proteinase from the tadpole tail of bullfrog, *Rana catesbeiana*. Comp Biochem Physiol 89B: 569–575

24 Okada S, Aikawa T (1986) Cathepsin D-like acid proteinase in the mantle of the marine mussel, *Mytilus edulis*. Comp Biochem Physiol 84B: 333–341

25 Oofusa K, Yoshizato K (1991) Biochemical and immunological characterization of collagenase in tissues of metamorphosing bullfrog tadpoles. Develop Growth and Differ 33: 329–339

26 Retzek H, Steyrer E, Sanders EJ, Nimpf J, Schneider WJ (1992) Molecular cloning and functional characterization of chicken cathepsin D, a key enzyme for yolk formation. DNA Cell Biol 11: 661–672

27 Rich DH (1986) Inhibitors of aspartic proteinase. In "Proteinase inhibitors" Ed by Barret AJ, Salvesen G, Elsevier Science Publishers BV., Amsterdam, pp 179–217

28 Runeberg-Roos P, Törmäkangas K, Östman A (1991) Primary structure of a barley-grain aspartic proteinase. Eur J Biochem 202: 1021–1027

29 Sakai J, Horiuchi S (1979) Characterization of cathepsin D in the regressing tadpole tail of bullfrog, *Rana catesbeiana*. Comp Biochem Physiol 62B: 269–273

30 Sapolsky AI, Woessner FJ (1972) Multiple forms of cathepsin D from bovine uterus. J Biol Chem 247: 2069–2076

31 Seshimo H, Ryuzaki M, Yoshizato K (1977) Specific inhibition of triiodothyonine-induced tadpole tail-fin regression by cathepsin D-inhibitor pepstatin. Develop Biol 59: 96–100

32 Shewale JG, Tang J (1984) Amino acid sequence of porcine spleen cathepsin D. Proc Natl Acad Sci USA 81: 3703–3707

33 Sogawa K, Fujii-Kuriyama Y, Mizukami Y, Ichihara Y, Takahashi K (1983) Primary structure of human pepsinogen gene. J Biol Chem 258: 5306–5311

34 Staros JV, Wright RW, Swingle DM (1986) Enhancement by N-hydroxysùlfosuccinimido of water soluble carbodiimide-mediated coupling reactions. Anal Biochem 156: 220–222

35 Takahashi T, Tang J (1983) Amino acid sequence of porcine spleen cathepsin D light chain. J Biol Chem 258: 6435–6443

36 Taylor AC, Kollros JJ (1946) Stages in the normal development of *Rana pipiens* larvae. Anal Rec 94: 7–23

37 Umezawa H, Aoyagi T, Morishima H, Matsuzaki M, Hamada M, Takeuchi T (1970) Pepstatin, a new pepsin inhibitor produced by actinomycetes. J Antibiot 23: 259–262

38 Weber R (1967) Biochemical and cellular aspects of tissue involution in development. Exp Biol Med 1: 63–76

39 Weber R (1977) Biochemical characteristics of tail atrophy during anuran metamorphosis. Colloq Int C N R S 226: 137–146

40 Wondrak EM, Louis JM, Mora PT, Oroszlan S (1991) Purification of HIV-1 wild type proteinase and characterization of proteolytically inactive HIV-1 proteinase mutants by pepstatin A affinity Chromatography. FEBS Lett 280: 347–350

41 Workman RJ, Burkitt DW (1979) Pepsin inhibition by a high

specific activity radioiodinated derivative of pepstatin. Arc Biochem Biophys 194: 157–164

42 Yamada H, Imoto T, Fujita K, Motomura M (1981) Selective modification of aspartic acid-101 in lysozyme by carbodiimide reaction. Biochemistry 20: 4836–4842

43 Yoshizato K (1989) Biochemistry and cell biology of amphibian metamorphosis with a special emphasis on the mechanism of removal of larval organs. In "International Review of Cytology" Academic Press, New York, 119: 97–149